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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
While bony metastases of prostate cancer are often osteoblastic, excessive bone resorption also occurs, which contributes to skeletal complications (e.g., pain, fractures). This research evaluates whether prostate cancer cells express the extracellular calcium ($Ca^{2+}{}_o$)-sensing receptor (CaSR) and whether the CaSR in bony metastases of prostate cancer participates in a vicious cycle involving CaSR-mediated secretion of the bone-resorbing cytokine, parathyroid hormone-related protein (PTHrP). The secreted PTHrP would promote further bone resorption, thereby increasing $Ca^{2+}{}_o$ locally and stimulating further PTHrP release. The project entails four tasks--namely showing that: (1) prostate cancer cells express the CaSR, (2) the CaSR mediates high $Ca^{2+}{}_o$-induced stimulation of PTHrP secretion, (3) the CaSR transactivates the EGF receptor, and (4) CaSR-stimulated PTHrP secretion from prostate cancer cells increases the severity of metastatic bone disease in vivo in mice. We have accomplished most of tasks 1 and 2, demonstrated that the CaSR likely transactivates the CaSR in task 3 and initiated the development of the stably transfected cell lines needed for the studies in task 4. These results support a role for the CaSR in a vicious cycle that increases the severity of bone resorption in vivo in humans.

14. SUBJECT TERMS:
CA^{2+}-sensing receptor, prostate cancer, bone metastases, PC-3 cells, EGF receptor, transactivation

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INTRODUCTION:

While prostate cancer research has generally emphasized the osteoblastic nature of prostate cancer metastases to bone, a wealth of recent data documents the nearly universal presence of excessive bone resorption as well, which contributes importantly to associated bone pain and fractures. The purpose of this research is to determine whether prostate cancer cells express the extracellular calcium (Ca^{2+}_o)-sensing receptor (CaSR) and whether the latter participates in a vicious cycle that promotes excessive bone resorption. This vicious cycle entails CaSR-mediated secretion of the bone-resorbing cytokine, parathyroid hormone-related protein (PTHrP), by skeletal metastases of prostate cancer. The secreted PTHrP would promote further bone resorption, which would increase the local concentration of Ca^{2+}_o, thereby stimulating further PTHrP release by the prostate cancer cells, and so forth. The scope of the work includes four specific aims: (1) to demonstrate that prostate cancer cells express the CaSR; (2) to prove that the CaSR mediates high Ca^{2+}_o-induced stimulation of PTHrP secretion in vitro; (3) to investigate whether the CaSR initiates a paracrine pathway leading to transactivation of the EGF receptor, which then produces EGFR-mediated activation of MAPK and, in turn, enhanced PTHrP production; and (4) to demonstrate that CaSR-mediated stimulation of PTHrP secretion from prostate cancer cells injected into the femora of nude mice contributes to the severity of metastatic bone disease by knocking out the receptor using a dominant negative CaSR construct. The progress that has been made in the tasks related to each of these specific aims relative to the time frames originally proposed for those tasks is detailed below.

BODY:

Task 1—To document that prostate cancer cell lines express the CaSR (months 1-18).

Subsequent to the submission of this grant proposal but prior to its funding, we largely completed the studies in task 1, which are described in detail in a publication of these studies (1). A reprint of this publication is appended to this report. These results of these studies are as follows:

We utilized both reverse transcriptase-polymerase chain reaction (RT-PCR) as well as Northern analysis to demonstrate that both the LnCaP and PC-3 prostate cancer cell lines express CaSR transcripts. The use of RT-PCR, with intron-spanning primers--to avoid the generation of PCR products of the same size arising from genomic DNA--amplified a product of the expected size, 480 bp, for having been derived from authentic CaSR transcript(s) [Figure 1 in ref. (1)]. In addition, Northern analysis using a CaSR-derived riboprobe and poly(A+) RNA derived from both LnCaP and PC-3 cells revealed a major transcript of 5.2 kb [Figure 1 in ref. (1)], similar in size to the major transcript in human parathyroid gland (2).

The use of immunocytochemistry with a polyclonal CaSR-specific antiserum demonstrated specific staining of both PC-3 and LnCaP cells as documented by its abolition following preabsorption of the antiserum with the specific peptide against which it was raised [Figure 2 in ref. 1)]. We also utilized Western blotting with the same antiserum to detect CaSR protein. Please note that in the interest of cost containment, we did not obtain original reprints but rather provide good quality Xeroxes of a downloaded version of the publication. Therefore, we apologize for the loss of resolution of certain figures (e.g., Northern blots and immunocytochemistry). The two prostate cancer cell lines exhibited immunoreactive bands of 160-170 kDa, comparable in size to bands identified in the positive controls—bovine parathyroid gland and CaSR-transfected human embryonic kidney (HEK293) cells [Figure 3 in ref. (1)]. The

immunoreactive bands of the sizes expected for the CaSR on Western analysis were specific as they were abolished following preincubation of the antiserum with the peptide against which it was raised.

Thus we have demonstrated that LnCaP as well as PC-3 cells express both CaSR transcript and protein. PC-3 cells will be utilized for the studies in task 4 aimed at demonstrating that knocking out the CaSR in these cells by transfection with a dominant negative CaSR construct will reduce the severity of osteolytic lesions in mice whose femora have been injected with PC-3 cells. Please note that while we originally proposed studies determining whether prostate cancer specimens removed at the time of prostatectomy expressed CaSR transcript(s) and protein, the contract for our grant expressly forbids the use of human anatomical substances. Should the parties reviewing this progress report wish these studies to be carried out, however, we would be pleased to carry them out during months 12-24.

Task 2—To show that the CaSR mediates the stimulation of PTHrP secretion from prostate cancer cell lines by high Ca^{2+}_o (months 6-24).

Our initial studies directed at documenting that the CaSR mediates the stimulatory effect of high Ca^{2+}_o on PTHrP secretion from PC-3 cells (1) utilized polycationic agents (e.g., neomycin and spermine) that are known to activate the cloned CaSR (3, 4). These two polycationic CaSR agonists were at least as efficacious as high Ca^{2+}_o in stimulating PTHrP secretion from PC-3 cells (Figure 4 in ref. (1)].

We then utilized a dominant negative CaSR construct (R185Q) to assess the CaSR's role in stimulating PTHrP secretion, since this would be the approach used to knock out the CaSR in PC-3 cells in Task 4 prior to injection of the cells into the femoral cavities of nude mice. Rather than generating stable transfectants with the dominant negative CaSR construct, however, we utilized infection with an adenoviral construct. Relative to vector-infected cells, those infected with the dominant negative CaSR showed substantial attenuation of the stimulation of PTHrP secretion by 1.5 and 3.5 mM Ca^{2+}_o--levels that might be anticipated to be encountered by bony metastases of prostate cancer cells close to sites of active bone resorption (5).

Task 3—To investigate whether the CaSR transactivates the EGFR in prostate cancer cells (months 6-24).

In addition to the studies accomplished in tasks 1 and 2, we have obtained initial evidence that the CaSR transactivates the EGFR as assessed by inhibition of high Ca^{2+}_o-stimulated PTHrP secretion by an inhibitor of the EGFR kinase (AG1478) but not by an inhibitor of the platelet-derived growth factor receptor (PDGFR) kinase (AG1296) (see Figure 1 in appendix). In addition, a neutralizing antibody to heparin-bound EGF, the form of EGF that we postulate transactivates the EGFR, produces a dose dependent inhibition of basal and high Ca^{2+}_o–stimulated PTHrP secretion at both low (0.5 mM) as well as high (3.0 and 7.5 mM) levels of Ca^{2+}_o (see Figure 2 in appendix). Furthermore, addition of exogenous EGF at 0.5 mM Ca^{2+}_o stimulated PTHrP secretion at 0.5 mM Ca^{2+}_o providing a positive control and showing that PC-3 cells express the EGFR (see Figure 3 in appendix). In addition to demonstrating suppression of PTHrP secretion by agents inhibiting the EGFR, we have also shown that inhibitors of two MAPK cascades--p38 MAPK and JNK—reduce PTHrP secretion (see Figure 2 in appendix), strongly suggesting that these two pathways contribute to high Ca^{2+}_o-stimulated PTHrP secretion.

Task 4—To show that knocking out the CaSR reduces the severity of bone resorption in the femora of nude mice injected with PC-3 cells (months 6-36).
We initiated during months 6-12 the development of PC-3 cells stably transfected with a dominant negative CaSR or with the corresponding vector. We have transfected PC-3 cells with a standard mammalian expression vector (pcDNA3) and subjected the transfected cells to selection with hygromycin. To date we have not yet been successful in obtaining individual, stably transfected PC-3 clones, in part because the cells grow very slowly at low density, but these studies are ongoing.

KEY RESEARCH ACCOMPLISHMENTS:
•Documented the presence of CaSR transcripts in PC-3 and LnCaP cells as assessed by RT-PCR and Northern analysis.
•Demonstrated the presence of CaSR protein in PC-3 and LnCaP cells as assessed by immunocytochemistry and Western analysis.
•Shown that polycationic CaSR agonists stimulate PTHrP secretion from PC-3 cells, consistent with the CaSR's involvement in mediating high Ca^{2+}_{o}–evoked PTHrP secretion.
•Documented reduction of high Ca^{2+}_{o}-stimulated PTHrP secretion from PC-3 cells by infection of the cells with a dominant CaSR construct, supporting the CaSR's mediatory role.
•Demonstrated that an inhibitor of the EGF receptor kinase reduces high Ca^{2+}_{o}-stimulated PTHrP secretion, consistent with the involvement of CaSR-mediated transactivation of the EGFR.
•Shown that a neutralizing antibody to HB-EGF reduces high Ca^{2+}_{o}–stimulated PTHrP secretion, providing further evidence that the CaSR transactivates the EGFR.
•Shown that high Ca^{2+}_{o} stimulates MAPK cascades at high Ca^{2+}_{o}, which, in turn, stimulate PTHrP secretion, as documented by the use of inhibitors of those pathways.

REPORTABLE OUTCOMES:
Although the studies in the following publication was completed after submission of the proposal for the present grant but prior to its being funded, it includes work that was proposed in tasks 1 and 2 and is listed for this reason.
Sanders JL, Chattopadhyay N, Kifor O, Yamaguchi T, Brown EM. Ca^{2+}_{o}-sensing receptor expression and PTHrP secretion on PC-3 human prostate cancer cells. Am J Physiol Endocrinol Metab 281:E1267-E1274, 2001.

CONCLUSIONS:
Our results to date support the major underling hypotheses driving this research, namely that the CaR mediates high Ca^{2+}_{o}-stimulated PTHrP secretion from PC-3 cells and could provide the basis for a "feed-forward" mechanism *in vivo* that would serve to aggravate the skeletal complications of prostate cancer metastatic to bone. The importance of this research lies in the implication that the CaR could serve as a therapeutic target for CaR antagonists that could diminish the severity of the skeletal complications of prostate cancer. Furthermore, it is possible that expression of the CaR in other cancers that metastasize to bone (e.g., breast cancer) could serve as the mediator of a similar "feed-forward" and thereby provide the basis for a novel therapy of cancers other than prostate cancer.

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APPENDICES:

Three graphs (Figures 1, 2 and 3—see text for details)
One publication (ref. (1) above].

Figure Legends

Figure 1: Effect of various protein kinase inhibitors on basal (0.5 mM) and high $Ca^{2+}{}_o$ (3 and 7.5 mM)-stimulated PTHrP secretion from PC-3 prostate cancer cells. The inhibitors are as follows: AG1478, EGF receptor kinase inhibitor; AG1296 platelet-derived growth factor (PDGF) receptor kinase inhibitor; PD98059, inhibitor of the ERK1/2 MAPK cascade; SB203580, inhibitor of p38 MAPK; SP600125, inhibitor of the JNK MAPK cascade. Note that the EGF receptor kinase inhibitor (but not the PDGF receptor kinase inhibitor) and the JNK inhibitor are the most effective inhibitors. See text for additional details.

Figure 2: A neutralizing antibody to heparin-bound EGF (HBEGF Ab) produces a dose-dependent inhibition of basal and high $Ca^{2+}{}_o$-stimulated PTHrP secretion from PC-3 cells—consistent with transactivation of the EGFR by release of endogenous HBEGF both under basal as well as high $Ca^{2+}{}_o$-stimulated conditions.

Figure 3: An antibody to HBEGF inhibits, and exogenous EGF stimulates, PTHrP secretion from PC-3 cells, consistent with a role for the EGF receptor and its transactivation in the control of PTHrP secretion by these cells.

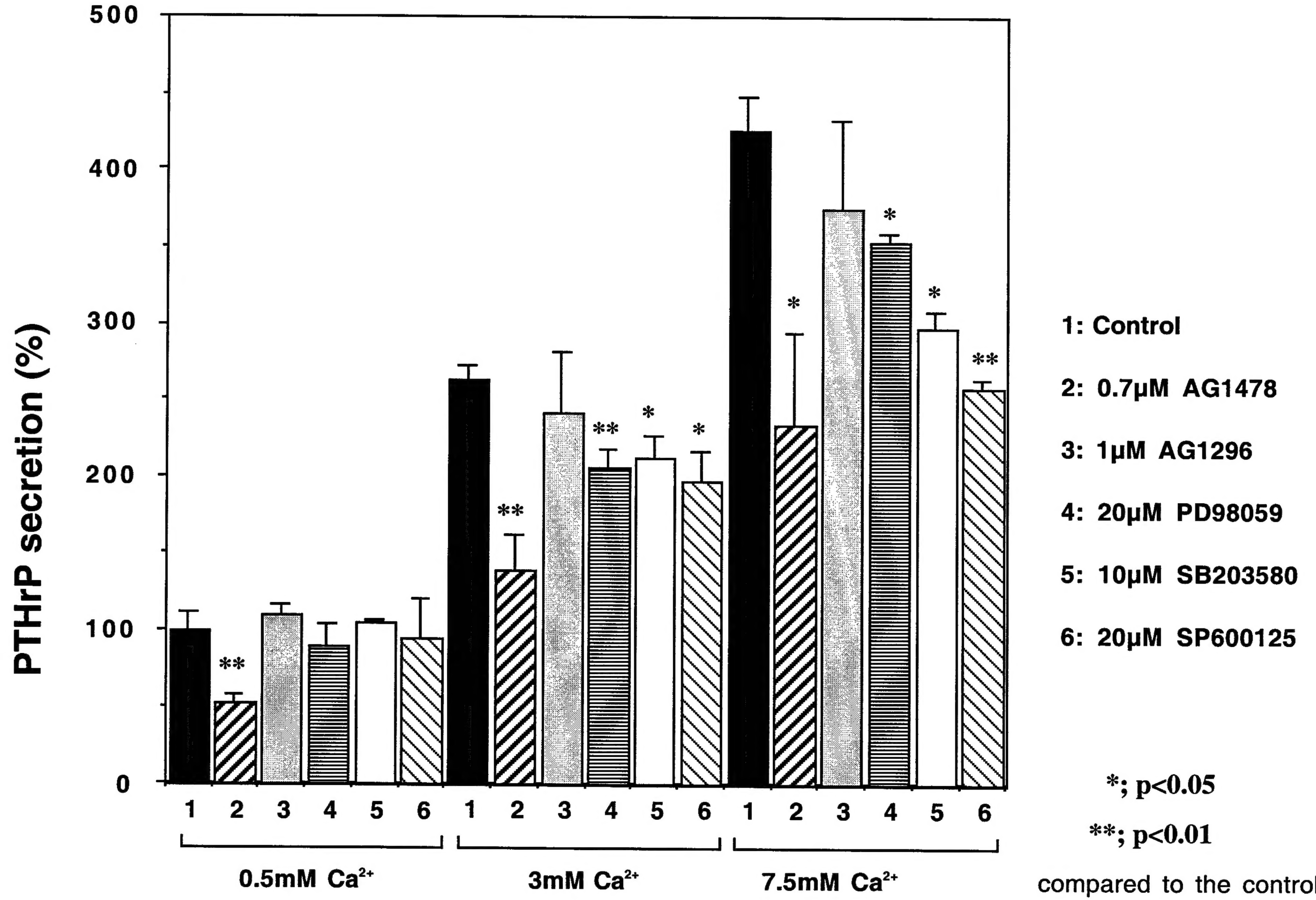
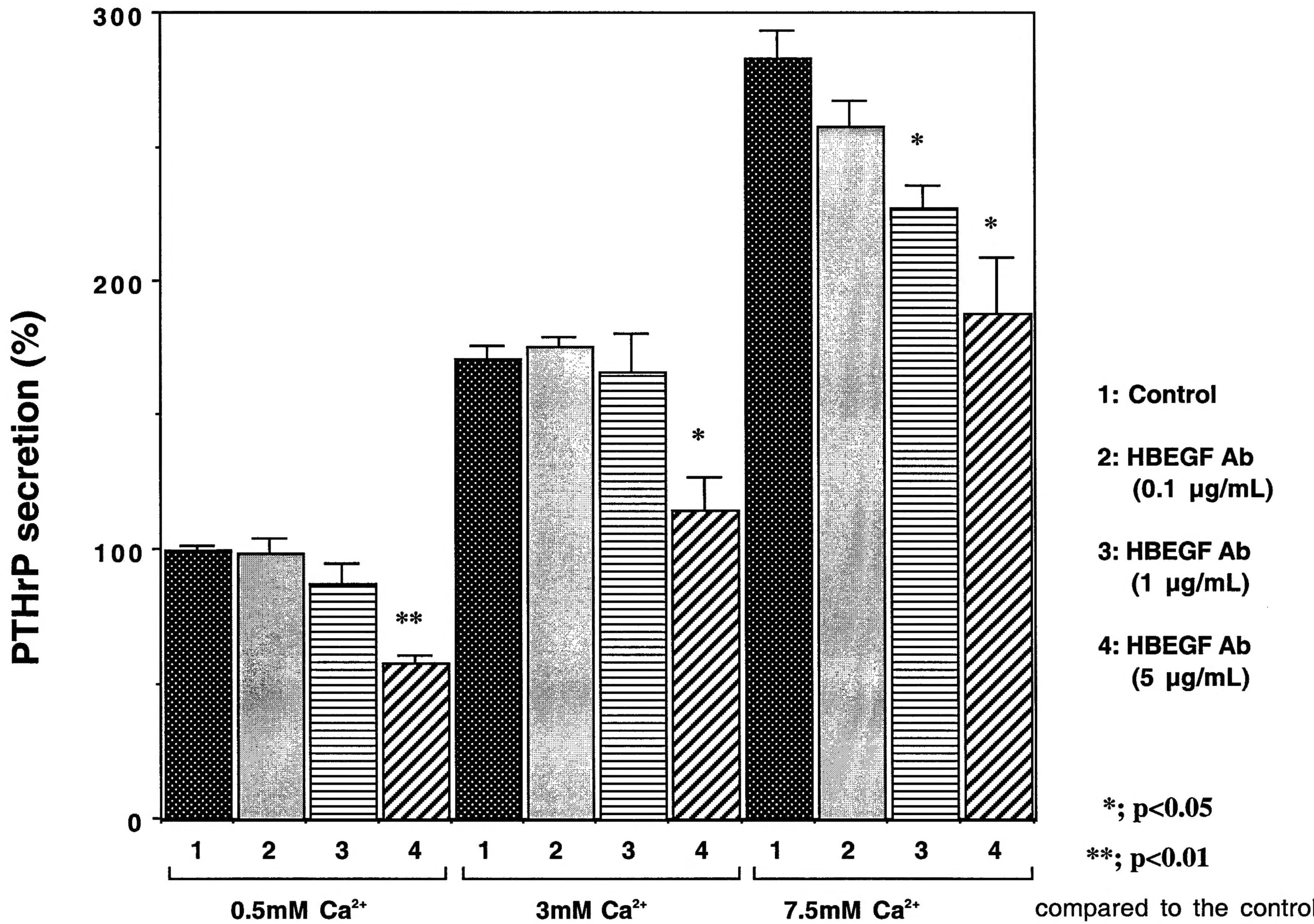

PTHrP secretion (%)
500
400
300
200
100
0
1 2 3 4 5 6
0.5mM Ca2+
3mM Ca2+
7.5mM Ca2+
*
**
1: Control
2: 0.7μM AG1478
3: 1μM AG1296
4: 20μM PD98059
5: 10μM SB203580
6: 20μM SP600125
*; p<0.05
**; p<0.01
compared to the control

Figure 1



PTHrP secretion (%)
300
200
100
0
**
*
*
*
1
2
3
4
1
2
3
4
1
2
3
4
0.5mM Ca2+
3mM Ca2+
7.5mM Ca2+
1: Control
2: HBEGF Ab (0.1 μg/mL)
3: HBEGF Ab (1 μg/mL)
4: HBEGF Ab (5 μg/mL)
*; p<0.05
**; p<0.01
compared to the control

Figure 2

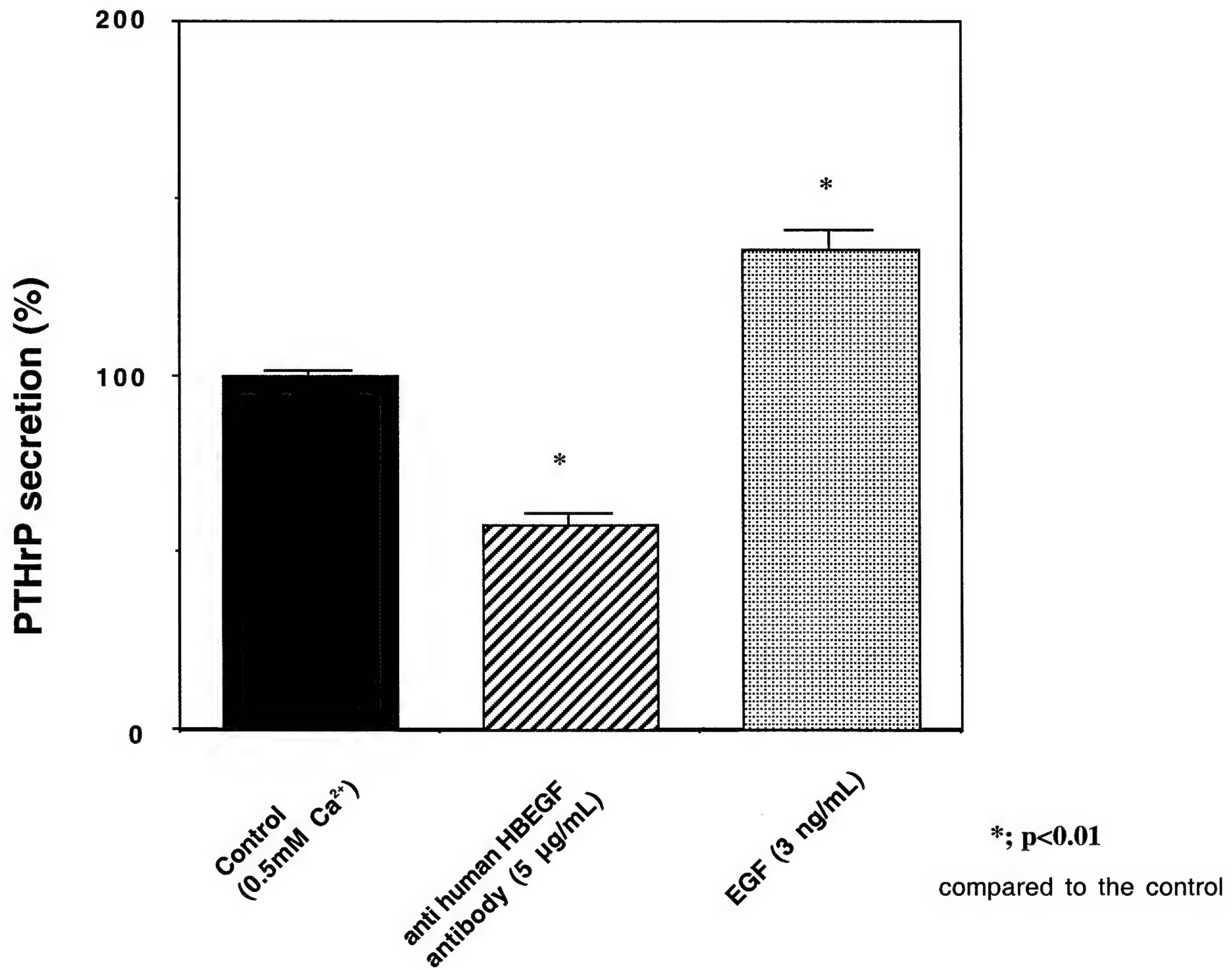

PTHrP secretion (%)
200
100
0
Control (0.5mM Ca2+)
anti human HBEGF antibody (5 μg/mL)
EGF (3 ng/mL)
*
*
*; p<0.01
compared to the control

Figure 3

Am J Physiol Endocrinol Metab
281: E1267–E1274, 2001.

Ca^{2+}-sensing receptor expression and PTHrP secretion in PC-3 human prostate cancer cells

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Sanders, Jennifer L., Naibedya Chattopadhyay, Olga Kifor, Toru Yamaguchi, and Edward M. Brown. Ca^{2+}-sensing receptor expression and PTHrP secretion in PC-3 human prostate cancer cells. *Am J Physiol Endocrinol Metab* 281: E1267–E1274, 2001.—Prostate cancer metastasizes frequently to bone. Elevated extracellular calcium concentrations ($[Ca^{2+}]_o$) stimulate parathyroid hormone-related protein (PTHrP) secretion from normal and malignant cells, potentially acting via the $[Ca^{2+}]_o$-sensing receptor (CaR). Because prostate cancers produce PTHrP, if high $[Ca^{2+}]_o$ stimulates PTHrP secretion via the CaR, this could initiate a mechanism whereby osteolysis caused by bony metastases of prostate cancer promotes further bone resorption. We investigated whether the prostate cancer cell lines LnCaP and PC-3 express the CaR and whether polycationic CaR agonists stimulate PTHrP release. Both PC-3 and LnCaP prostate cancer cell lines expressed bona fide CaR transcripts by Northern analysis and RT-PCR and CaR protein by immunocytochemistry and Western analysis. The polycationic CaR agonists $[Ca^{2+}]_o$, neomycin, and spermine each concentration dependently stimulated PTHrP secretion from PC-3 cells, as measured by immunoradiometric assay, with maximal, 3.2-, 3.6-, and 4.2-fold increases, respectively. In addition, adenovirus-mediated infection of PC-3 cells with a dominant negative CaR construct attenuated high $[Ca^{2+}]_o$-evoked PTHrP secretion, further supporting the CaR's mediatory role in this process. Finally, pretreating PC-3 cells with transforming growth factor (TGF)-β_1 augmented both basal and high $[Ca^{2+}]_o$-stimulated PTHrP secretion. Thus, in PTHrP-secreting prostate cancers metastatic to bone, the CaR could initiate a vicious cycle, whereby PTHrP-induced bone resorption releases $[Ca^{2+}]_o$ and TGF-β stored within bone, further increasing PTHrP release and osteolysis.

parathyroid hormone-related protein; ion-sensing receptor; osteolysis; prostate cancer; LnCaP cells; skeletal metastases

PROSTATE CANCER IS A COMMON CANCER and the second leading cause of cancer death in men (4). A substantial percentage of elderly men have microscopic prostate cancers, but these small lesions usually remain localized to the prostate and never come to clinical attention. Nevertheless, skeletal complications of prostate cancer are a difficult clinical problem, causing disabling pain and other complications such as fractures (10). Radiation, hormonal manipulations, and/or chemotherapy offer palliation but, unfortunately, little hope of cure for skeletal metastases of prostate cancer. Therefore, further understanding of the biology of prostate cancer metastatic to bone and the development of improved therapies of skeletal metastases and their complications are important goals of prostate cancer research.

Recent studies have shown that parathyroid hormone (PTH)-related protein (PTHrP) is a central mediator of malignancy-associated hypercalcemia and osteolysis. In addition to causing most cases of humoral hypercalcemia of malignancy, where skeletal metastases are absent, PTHrP, originally isolated from renal, lung, and breast cancers (7, 37, 39), is the biological mediator in ~70% of cases of malignant osteolysis with or without hypercalcemia, particularly that caused by common epithelial cancers [i.e., breast (16)]. Although prostate cancers metastatic to bone generally cause osteoblastic lesions, substantial increases in bone resorption also occur in this setting, as assessed by biochemical markers (10, 24, 40). Indeed, markers of bone resorption can be higher in patients with metastatic prostate cancer than in those with skeletal metastases of breast cancer (10). Prostate cancers often express more PTHrP than normal prostate epithelial cells (1, 25), suggesting that PTHrP could contribute to the increased bone resorption (10) in patients with prostate cancer metastatic to bone (1, 25, 38). PTHrP secreted by prostate cancer cells could then activate osteoclasts and potentially contribute to skeletal invasiveness, bone pain, and/or pathological fractures. Therefore, further understanding of the factors regulating the production and secretion of PTHrP by prostate cancer cells could elucidate the mechanisms underlying the excessive bone resorption associated with this tumor and potentially provide clues to novel therapeutic strategies.

The extracellular calcium ($[Ca^{2+}]_o$)-sensing receptor (CaR) is a G protein-coupled cell surface receptor that is a central element in $[Ca^{2+}]_o$ homeostasis (6). In

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parathyroid cells, high $[Ca^{2+}]_o$, by activating the CaR, inhibits PTH secretion and parathyroid cellular proliferation (6), whereas in the kidney, stimulating the receptor reduces renal tubular Ca^{2+} reabsorption (20). Physiological proof of the CaR's key roles in $[Ca^{2+}]_o$ homeostasis has come from the identification of hyper- and hypocalcemic disorders caused by inactivating or activating CaR mutations (5), respectively, and from mice with targeted disruption of the CaR gene (23).

In addition to inhibiting PTH release from parathyroid cells, the CaR stimulates the secretion of calcitonin from C cells (12, 14) and of ACTH from AtT-20 cells (11). Furthermore, several studies have shown that high $[Ca^{2+}]_o$ can stimulate PTHrP release from normal keratinocytes (22), normal cervical epithelial cells (28), oral squamous cancer cells (31), and JEG-3 cells (21), suggesting that the CaR could be the mediator of high $[Ca^{2+}]_o$-evoked PTHrP release from both normal and malignant cells. In the case of PTHrP-secreting prostate cancers metastatic to bone, this CaR-mediated action could create an inappropriate "feed-forward" stimulation of PTHrP secretion, causing release of Ca^{2+} from bone that would stimulate further PTHrP secretion and promote worsening bone resorption. Moreover, interrupting high $[Ca^{2+}]_o$-evoked, CaR-mediated PTHrP secretion from prostate cancer cells [e.g., with a CaR antagonist (15)] could potentially be of substantial clinical benefit in this setting. The goals of the present study, therefore, were to determine whether two commonly employed prostate cancer cell lines, LnCaP and PC-3, express the CaR, and if so, whether this receptor participates in the regulation of PTHrP secretion. Our results suggest that the CaR is expressed in and likely mediates high $[Ca^{2+}]_o$-induced PTHrP secretion from PC-3 cells. Furthermore, transforming growth factor (TGF)-β_1, stimulates PTHrP secretion from PC-3 cells synergistically with high $[Ca^{2+}]_o$, suggesting that release of this growth factor, along with calcium, during PTHrP-induced bone resorption could contribute to a feed-forward mechanism in which PTHrP-mediated osteolysis associated with prostate cancers metastatic to bone begets worsening osteolysis.

MATERIALS AND METHODS

Cell culture. The LnCaP and PC-3 human prostate cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured in RPMI-1640 medium supplemented with 10% FCS and 100 U/ml penicillin-100 μg/ml streptomycin. The cells were grown at 37°C in a humidified 5% CO_2 atmosphere and were passaged every 5–7 days with the use of either 0.25% trypsin-0.53 mM EDTA (LnCaP cells) or 0.05% trypsin-0.53 mM EDTA (PC-3 cells). All cell culture reagents were purchased from GIBCO-BRL (Grand Island, NY), with the exception of FCS, which was obtained from Gemini Bio-Products, (Calabasas, CA).

Northern blotting. Total RNA was prepared using TRIzol reagent (GIBCO-BRL). Northern blot analysis was performed on 7.5 μg of poly(A^+) RNA obtained using oligo-dT cellulose chromatography of total RNA (8). Poly(A^+)-enriched RNA samples were denatured and electrophoresed in 2.2 M formaldehyde-1% agarose gels along with a 0.24- to 9.5-kb RNA ladder (GIBCO-BRL) and transferred overnight to nylon membranes (Duralon; Stratagene, La Jolla, CA). A ^{32}P-labeled riboprobe corresponding to nucleotides 1745–2230 of the human parathyroid CaR cDNA was synthesized with the MAXIscript T_3 kit (Pharmacia Biotech, Piscataway, NJ) with the use of T_3 RNA polymerase and [^{32}P]UTP. Nylon membranes were then prehybridized, hybridized overnight with the labeled cRNA probe (2×10^6 cpm/ml), and washed at high stringency for 30 min as described previously (35). Membranes were sealed in plastic bags and exposed to a PhosphorImager screen. The screens were analyzed on a Molecular Dynamics PhosphorImager (Sunnyvale, CA) with the ImageQuant program.

RT-PCR. Total RNA (3–5 μg) was used for the synthesis of first-strand cDNA (cDNA synthesis kit, GIBCO-BRL). The resultant first-strand cDNA was used for PCR, which was performed in a buffer containing (in mM): 20 Tris·HCl, pH 8.4, 50 KCl, 1.8 $MgCl_2$, and 0.2 dNTP and 0.4 μM forward primer, 0.4 μM reverse primer, and 1 μl ELONGASE enzyme mix (a *Taq/Pyrococcus species GB-D* DNA polymerase mixture; GIBCO-BRL). Human parathyroid CaR sense primer 5′-CGGGGTACCTTAAGCACCTACGGCATCTAA-3′ and antisense primer 5′-GCTCTAGAGTTAACGCGATCCCAAA-GGGCTC-3′, which are intron spanning, were used for the reactions. To perform "hot start" PCR, the enzyme mixture was added during the initial 3-min denaturation and was followed by 35 cycles of amplification (30-s denaturation at 94°C, 30-s annealing at 47°C, and 1-min extension at 72°C). The reaction was completed with an additional 10-min incubation at 72°C to allow completion of extension. PCR products were fractionated on 1.5% agarose gels. PCR products in the reaction mixture were purified using the QIAquick PCR purification kit (Qiagen, Santa Clarita, CA) and were subjected to bidirectional sequencing by employing the same primer pairs used for PCR by means of an automated sequencer (AB377; Applied Biosystems, Foster City, CA) as previously described (35).

Immunocytochemistry. A CaR-specific polyclonal antiserum (4637) was generously provided by Drs. Forrest Fuller and Karen Krapcho of NPS Pharmaceuticals. This antiserum was raised against a peptide corresponding to amino acids 345–359 of the bovine CaR, which is identical to the corresponding peptide in the human CaR and resides within the predicted amino-terminal extracellular domain of the CaR. The antiserum was subjected to further purification by means of an affinity column conjugated with the FF-7 peptide (27), and the affinity-purified antiserum was used for immunocytochemistry and Western blot analysis as described in the following paragraphs. The specificity of the antiserum for the CaR is documented in RESULTS by the use of suitable positive and negative controls.

For immunocytochemistry, prostate cancer cells were grown on glass coverslips (27), fixed for 5 min with 4% formaldehyde, and then treated for 10 min with peroxidase blocking reagent (DAKO, Carpenteria, CA) to inhibit endogenous peroxidases. After washing with PBS, the cells were blocked for 30 min with PBS containing 1% BSA. The cells were then incubated overnight at 4°C with the 4637 antiserum (5 μg/ml in blocking solution). Negative controls were carried out by incubating cells treated in an otherwise identical manner with the same concentration of 4637 antiserum that had been preabsorbed with 10 μg/ml of the FF-7 peptide. The cells were then washed, incubated with peroxidase-conjugated goat anti-rabbit IgG (1:100; Sigma Chemical, St. Louis, MO) and washed again, and the color reaction was developed using the DAKO AEC substrate system (DAKO) as

before (27). The cells were observed by light microscopy and photographed at ×400 magnification.

Western Blotting. For Western blotting, confluent monolayers of LnCaP and PC-3 cells in 6-well plates were rinsed with ice-cold PBS and scraped on ice into lysis buffer containing 10 mM Tris·HCl, pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.25 M sucrose, 1% Triton X-100, 1 mM dithiothreitol, and a cocktail of protease inhibitors (10 μg/ml each of aprotinin, leupeptin, and calpain inhibitor, as well as 100 μg/ml of Pefabloc) (26). The cells were then passed though a 22-gauge needle 10 times. Nuclei and other cellular debris were removed by low-speed centrifugation (1,000 *g* for 10 min), and the resultant total cellular lysate in the supernatant was used either directly for SDS-PAGE or stored at −80°C. Bovine parathyroid cells, CaR-transfected HEK-293 cells (designated HEKCaR), or nontransfected HEK-293 cells, included as positive (parathyroid and HEKCaR) and negative controls (nontransfected HEK-293 cells), were harvested according to the same protocol.

Immunoblot analyses were performed essentially as described before (26, 27). Aliquots of 20–40 μg of protein were mixed with an equal volume of 2× SDS-Laemmli gel loading buffer containing 100 mM dithiothreitol, incubated at 37°C for 15 min, and resolved electrophoretically on linear 3–10% gradient gels. The separated proteins were then transferred to nitrocellulose blots (Schleicher & Schuel, Keene, NH) and incubated with blocking solution (PBS with 0.25% Triton X-100 and 5% dry milk) for 1 h at room temperature. The blots were incubated overnight at 4°C with affinity-purified anti-CaR polyclonal antiserum 4637 at 1 μg/ml with or without preincubation with 2 μg/ml of the FF-7 peptide in blocking solution with 1% dry milk. The blots were subsequently washed, incubated with a 1:2,000 dilution of horseradish peroxidase-coupled goat anti-rabbit IgG in blocking solution, and washed five times again, and protein bands were detected using an enhanced chemiluminescence (ECL) system (Renaissance Kit, Du Pont-NEN).

Adenoviral infection of dominant negative CaR into PC-3 cells. Confluent PC-3 cells were scraped, dispersed by repeated pipetting, and then seeded in 24-well plates (~2.5 × 10^3 cells/well). Approximately 10,000 infective particles containing dominant negative CaR (R185Q) or empty vector as a negative control were added to each well at the time the cells were seeded in growth medium. The cells were then cultured for 48 h, washed with PBS, and then incubated with DMEM (containing 0.2% BSA and 0.5 mM $[Ca^{2+}]_o$) for 2 h. Additional calcium was then added to the wells as needed to achieve the final concentrations indicated in RESULTS, and the cells were incubated overnight. At the end of the incubation, conditioned medium was collected and subjected to PTHrP assay as described in *PTHrP secretion studies*. The data were normalized to the amount of protein in each well. Experiments were carried out using triplicate wells for each level of $[Ca^{2+}]_o$.

PTHrP secretion studies. For studies on the effects of various CaR agonists on PTHrP secretion, PC-3 cells were seeded in 96-well plates (5,000 cells/well) in 0.15 ml of *medium A* (RPMI-1640 supplemented with 10% FCS and 100 U/ml penicillin-100 μg/ml streptomycin). After 72 h, *medium A* was carefully removed, and the subconfluent cells in each well were rinsed once with 0.15 ml of *medium B* [calcium-free DMEM (GIBCO-BRL) supplemented with 4 mM L-glutamine, 2% FCS, 100 U/ml penicillin-100 μg/ml streptomycin, and 0.5 mM $CaCl_2$]. *Medium B* alone or *medium B* supplemented with either additional $CaCl_2$ (to final concentrations of 1, 3, 5, 7.5, or 10 mM) or the polycationic CaR agonists neomycin (100 or 300 μM) or spermine (2 mM) was then added to each well (0.275 ml/well). Six hours later, the conditioned medium was removed for determination of PTHrP content. Triplicate incubations were performed for each treatment, and each experiment was carried out at least twice.

For studies on the effects of pretreatment with TGF-β_1 on PTHrP secretion, PC-3 cells were seeded as described earlier. After 48 h, *medium A* was carefully removed from each well, and 0.15 ml of *medium C* (calcium-free DMEM supplemented with 4 mM L-glutamine, 0.2% BSA, 100 U/ml penicillin-100 μg/ml streptomycin, and 0.5 mM $CaCl_2$) containing 0, 0.2, 1, or 5 ng/ml TGF-β_1 was added to each well. Twenty-four hours later, this "pretreatment" medium was removed from each well, the cells were rinsed once with 0.15 ml/well *medium B*, and then *medium B* alone, or *medium B* supplemented with additional $CaCl_2$ (to final concentrations of 3, 5, 7.5, or 10 mM) was added to each well (0.275 ml/well). Six to twenty-four hours later, the conditioned medium was removed for determination of PTHrP content. Triplicate incubations were performed for each treatment, and each experiment was carried out at least twice.

PTHrP was measured in PC-3 cell-conditioned medium by means of a two-site immunoradiometric assay (IRMA; Nichols Institute Diagnostics, San Juan Capistrano, CA) that detects PTHrP-(1–72) and has a sensitivity of 0.3 pmol/l (35). PTHrP assays were initiated immediately after removal of the conditioned medium from the cell cultures to minimize the loss of PTHrP that occurs with freeze thawing or other manipulations. PTHrP concentrations were calculated from a standard curve generated by adding recombinant PTHrP-(1–86) to the treatment medium employed in this study (i.e., unconditioned *medium B*). $CaCl_2$ and the additional polycationic CaR agonists (neomycin, spermine) employed in these experiments had no effects in the PTHrP assay when added in the absence of PC-3 cell-conditioned medium.

To ensure that the CaR agonists employed in the PTHrP studies had no significant effects on cell number or viability over the 6-h treatment period, we employed the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (19), in which only viable cells convert water-soluble MTT to insoluble formazan crystals, as described previously (35).

Statistical analyses. A minimum of two independent PTHrP secretion experiments were performed for each of the PTHrP secretion studies described earlier. Results are presented as means ± SE for three determinations. Data were analyzed by analysis of variance followed by Fisher's protected least significant difference test. For all statistical tests, a P value <0.05 was considered to indicate a statistically significant result.

RESULTS

Detection of CaR mRNA in LnCaP and PC-3 cells by Northern analysis and RT-PCR. Northern blot analysis carried out using a CaR-specific riboprobe on poly(A^+) RNA isolated from LnCaP and PC-3 cells revealed a major transcript of ~5.2 kb (Fig. 1*A*). This transcript is similar in size to a major CaR transcript in human parathyroid gland (13). RT-PCR performed with intron-spanning primers specific for the human CaR amplified a product of the expected size, 480 bp, for a CaR-derived product in both LnCaP (Fig. 1*B*, *lane 2*) and PC-3 cells (Fig. 1*B*, *lane 3*). DNA sequence analysis of the PCR products revealed >99% sequence identity with the corresponding region of the human

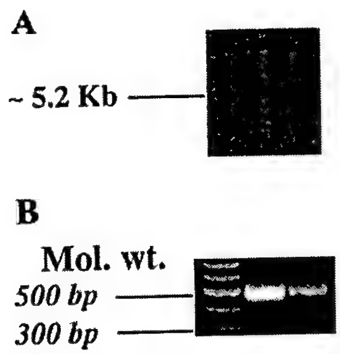

Fig. 1. *A*: Northern blot analysis of extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$)-sensing receptor (CaR) transcripts in the PC-3 and LnCaP prostate cancer cell lines. Northern analysis was performed on poly(A^+) RNA isolated from the LnCaP (*lane 1*) and PC-3 prostate cancer cell lines (*lane 2*), as described in MATERIALS AND METHODS, using a human CaR-specific riboprobe. *B*: expression of CaR transcripts as assessed by RT-PCR using CaR-specific primers in PC-3 and LnCaP cells. RT-PCR was performed on cDNA prepared from the same sample of RNA extracted from LnCaP cells (*lane 2*) or PC-3 cells (*lane 3*), as described in MATERIALS AND METHODS, using an intron-spanning primer pair specific for the human CaR. A 480-bp amplified fragment is indicative of a product arising from authentic CaR-derived transcript(s). *Lane 1* shows a DNA ladder for size comparison. No such product was apparent when cDNA was replaced with water or the reverse transcriptase was omitted from the RT reactions (not shown).

parathyroid CaR cDNA (not shown). These results indicate that the PCR products derived from both PC-3 and LnCaP cells were amplified from authentic CaR transcript(s).

Detection of CaR protein in LnCaP and PC-3 cells by immunocytochemistry and Western analysis. Immunocytochemistry with an anti-CaR antiserum (4637) revealed moderate CaR staining in both LnCaP (Fig. 2*A*) and PC-3 (Fig. 2*B*) prostate cancer cells. Staining was eliminated by preincubating the CaR antiserum with the specific peptide (FF-7) against which it was raised (Fig. 2, *C* and *D*). Considerable intracellular CaR immunoreactivity could be observed in these cells, as in breast cancer (35) and bone cells (43, 44), which express considerably less CaR protein than do parathyroid cells (26), where the CaR displays a predominantly rim-like pattern of cell surface expression. Western blot analyses of proteins isolated from total cellular lysates of LnCaP or PC-3 cells by use of the 4637 antiserum were compared with those obtained using protein preparations from HEKCaR and bovine parathyroid cells as positive controls and nontransfected HEK-293 cells as a negative control (Fig. 3, *A* and *C*). Although the level of CaR protein expression in HEKCaR cells was much higher than the level in LnCaP and PC-3 cells (Fig. 3*A*), the immunoreactive bands in the two prostate cancer cell lines of ~160–170 kDa are comparable in size to those of bands present in the positive controls (Fig. 3, *A* and *C*). The specificity of these 160- to 170-kDa CaR-immunoreactive bands in proteins from the prostate cancer cell lines was confirmed by the marked reductions in their intensities after preabsorption of the antiserum with the peptide against which it was raised, although nonspecific bands at lower molecular masses were not abolished by the preabsorption procedure (Fig. 3*B*).

Figure 3, *C* and *D* further documents the specificity of this antiserum for the CaR by comparing the pattern of CaR-immunoreactive bands recognized by antiserum 4637 in proteins prepared from HEKCaR cells, bovine parathyroid cells, and nontransfected HEK-293 cells. There are similar patterns of bands in HEKCaR and parathyroid cells, corresponding to various glycosylated and nonglycosylated forms of CaR monomers and dimers (3, 42), but no CaR-specific immunoreactivity in nontransfected HEK-293 cells, which do not express the CaR endogenously. Figure 3*C* also shows more clearly the sizes of the immunoreactive bands in HEKCaR cells than does the overexposed lane showing these bands in Fig. 3*A*.

Effect of CaR agonists, TGF-β_1, and dominant negative CaR on PTHrP secretion. To determine whether CaR agonists modulate PTHrP secretion from PC-3 cells, the cells were treated with varying levels of $[Ca^{2+}]_o$ (0.5, 1, 3, 5, 7.5, or 10 mM), neomycin (100 or 300 μM in 0.5 mM $[Ca^{2+}]_o$), or spermine (2 mM in 0.5 mM $[Ca^{2+}]_o$), and PTHrP in the conditioned medium was determined by IRMA. PC-3 cells produce a readily measurable amount of PTHrP at 0.5 mM $[Ca^{2+}]_o$. Higher levels of $[Ca^{2+}]_o$ stimulated PTHrP secretion in a dose-dependent manner (Fig. 4*A*). At 1, 3, and 5 mM $[Ca^{2+}]_o$, PTHrP secretion was increased 1.2-, 1.5-, and 1.8-fold, respectively, compared with that observed at

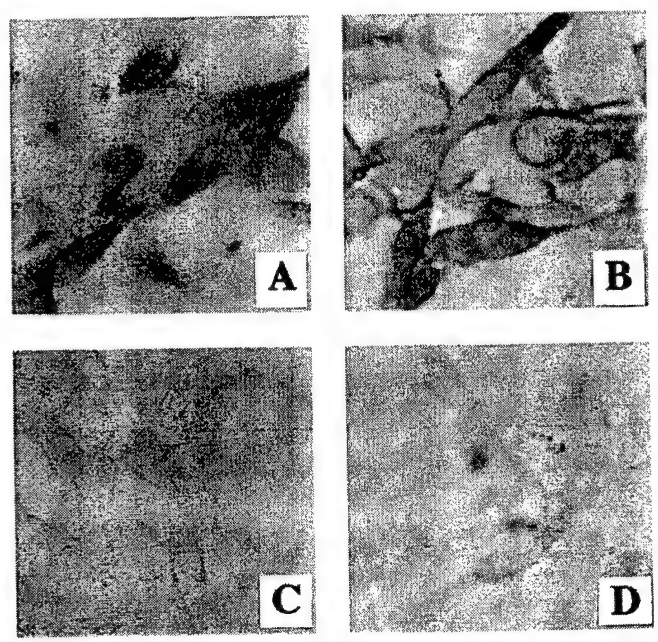

Fig. 2. Expression of CaR protein as assessed by immunocytochemistry using CaR-specific polyclonal antiserum 4637 in PC-3 and LnCaP cells. Immunocytochemistry, carried out using anti-CaR antiserum 4637 as described in MATERIALS AND METHODS, revealed readily apparent immunostaining of both cell lines, LnCaP cells (*A*) and PC-3 cells (*B*), which was eliminated by preincubating the CaR antiserum with the peptide FF-7 against which it was raised (*C*: LnCaP cells; *D*: PC-3 cells) (×400).

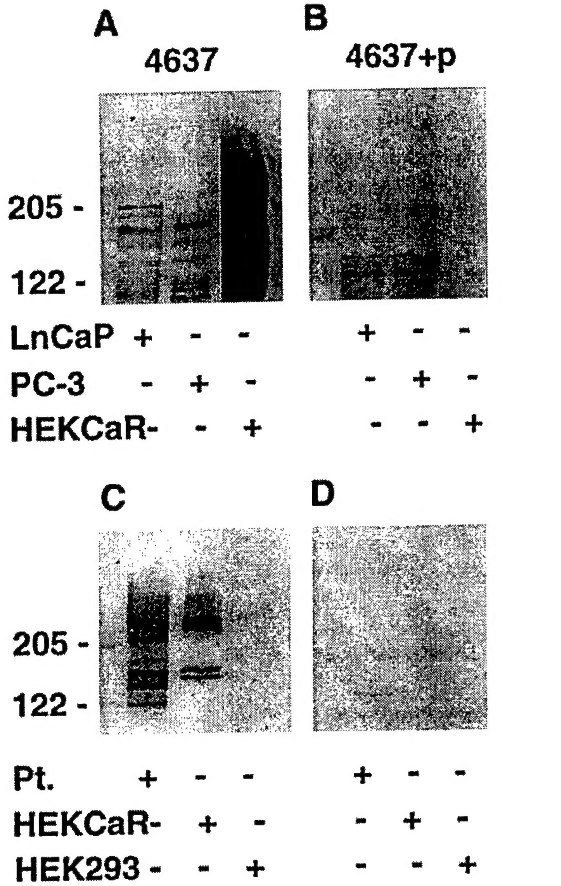

Fig. 3. *A* and *B*: expression of CaR protein as assessed by Western blot analysis using CaR-specific polyclonal antiserum 4637 in PC-3 and LnCaP cells. Western blot analyses of CaR proteins in whole cell lysates isolated from PC-3 or LnCaP prostate cancer cells, or from CaR-tranfected HEK-293 (HEKCaR) cells as a positive control, were carried out as described in MATERIALS AND METHODS. Each protein sample, 20 μg for HEKCaR cells (*right* lanes) and 40 μg for LnCaP and PC-3 cells (*left* and *middle* lanes, respectively), was subjected to SDS-PAGE. *A*: CaR-specific antiserum 4637 was used as described in MATERIALS AND METHODS to identify expression of CaR protein in the resultant blots as indicated in the figure. *B*: results observed when the antiserum was preabsorbed with the peptide against which it was raised. *C* and *D*: Western blots of proteins in crude membrane preparations from bovine parathyroid (*lane 1*), HEKCaR (*lane 2*), and nontransfected HEK-293 cells (*lane 3*) using anti-CaR antiserum 4637. *C*: crude plasma membrane proteins were prepared, SDS-PAGE was carried out, and Western blotting was performed as described in MATERIALS AND METHODS. *D*: results observed when the antiserum was preabsorbed with the peptide against which it was raised. The Western blots shown in *A-D* are representative of ≥2 such blots for each cell type.

0.5 mM $[Ca^{2+}]_o$. $[Ca^{2+}]_o$ at 7.5 and 10 mM evoked more substantial increases in PTHrP secretion (3.0- and 3.2-fold, respectively). The polycationic CaR agonists neomycin and spermine also elicited robust secretory responses: 100 and 300 μM neomycin increased PTHrP secretion 3.4- and 3.6-fold, respectively, relative to that at 0.5 mM $[Ca^{2+}]_o$, whereas 2 mM spermine induced a 4.6-fold increase in secretion.

Because TGF-β stimulates PTHrP secretion from some cancer cell lines [e.g., the MDA-MB-231 breast

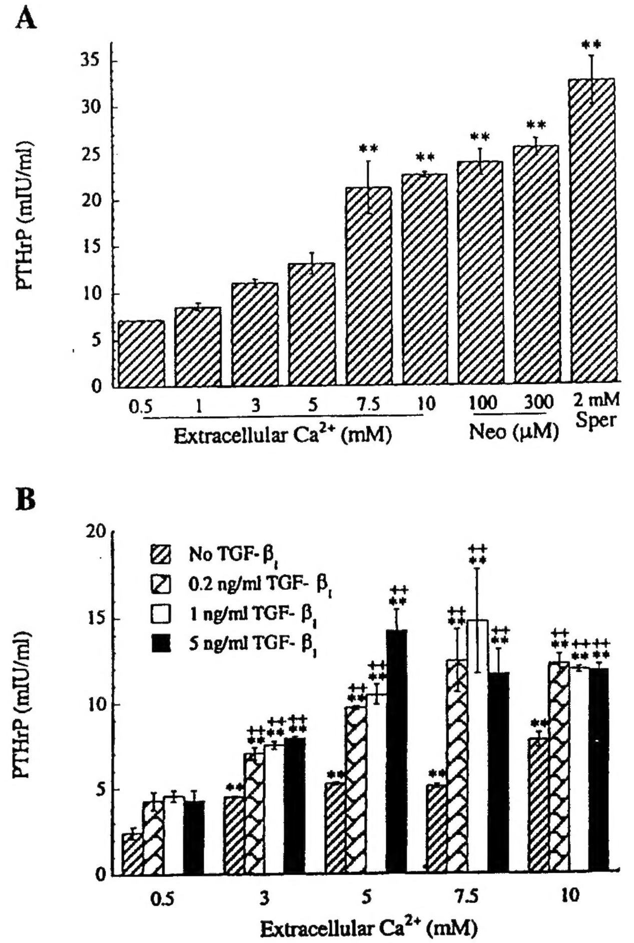

Fig. 4. *A*: effect of elevated levels of $[Ca^{2+}]_o$ and the polycationic CaR agonists neomycin (Neo) and spermine (Sper) on secretion of parathyroid hormone-related peptide (PTHrP) from PC-3 cells. Cells were treated for 6 h with CaR agonists, and the conditioned media were removed for determination of PTHrP released during the incubation, as described in MATERIALS AND METHODS. There was no difference in the MTT colorimetric assay for cells treated with different concentrations of CaR agonists (not shown), suggesting that there was no effect of these agents on cell number or viability, and results for PTHrP secretion are normalized to the MTT value for that well. There was a statistically significant stimulation of PTHrP secretion at ≥7.5 mM $[Ca^{2+}]_o$, as well as in the presence of neomycin or spermine ($P < 0.01$; $n = 3$) relative to that observed at 0.5 mM $[Ca^{2+}]_o$. Essentially identical results were observed in another experiment carried out using the identical experimental protocol. *B*: effect of pretreatment with transforming growth factor (TGF)-β_1 on high $[Ca^{2+}]_o$-stimulated PTHrP secretion from PC-3 cells. Cells were pretreated overnight with 0.2, 1, or 5 ng/ml of TGF-β_1, as described in MATERIALS AND METHODS, and then incubated for 6 h with the indicated levels of $[Ca^{2+}]_o$. PTHrP in the conditioned medium was then determined by immunoradiometric assay as in *A*, and the results for PTHrP secretion were normalized to the MTT value for that well. PTHrP secretion was statistically significantly stimulated at ≥3 mM $[Ca^{2+}]_o$ (**$P < 0.01$ vs. 0.5 mM $[Ca^{2+}]_o$ alone, $n = 3$) and with all concentrations of TGF-β_1 (**$P < 0.01$ vs. 0.5 mM $[Ca^{2+}]_o$ alone, $n = 3$; ++$P < 0.01$ vs. no TGF-β_1, $n = 3$) relative to the respective basal values at 0.5 mM $[Ca^{2+}]_o$. Essentially identical results were observed in another experiment carried out using the identical experimental protocol.

cancer cell line (35)], we examined the possibility that there might be an interaction between TGF-β and $[Ca^{2+}]_o$ on PTHrP secretion in PC-3 cells. When PC-3 cells were pretreated for 24 h with TGF-β_1, a substantial dose-dependent increase in both basal (i.e., at 0.5 mM $[Ca^{2+}]_o$) and high $[Ca^{2+}]_o$-stimulated PTHrP secretion was observed (Fig. 4*B*). Neither $[Ca^{2+}]_o$, neomycin, nor TGF-β_1 had any significant effect on the MTT values obtained from the PC-3 cells in this study, and the results of the MTT assay were employed to normalize the PTHrP released in each well.

To provide more definitive evidence that the CaR mediates high $[Ca^{2+}]_o$-evoked PTHrP secretion, we examined the effect of adenovirus-mediated infection of PC-3 cells with a dominant negative CaR construct (2) on $[Ca^{2+}]_o$-stimulated PTHrP secretion. Figure 5 shows that pretreatment of PC-3 cells with an adenoviral vector encoding the dominant negative CaR construct R185Q right-shifts the stimulation of PTHrP secretion by high $[Ca^{2+}]_o$ and attenuates the response observed at 10 mM $[Ca^{2+}]_o$ relative to the secretory response observed with PC-3 cells infected with a control adenoviral vector.

DISCUSSION

The purpose of this study was to determine whether the LnCaP and PC-3 human prostate cancer cell lines express the CaR, and if so, whether CaR agonists modulate PTHrP secretion from them. CaR expression was detected in LnCaP and PC-3 cells by both nucleotide- and protein-based approaches. Northern analysis performed on poly(A^+) RNA from each of the two cell lines revealed a 5.2-kb CaR transcript (Fig. 1*A*). This transcript is similar in size to one of the predominant CaR transcripts observed in human parathyroid cells (13). Authentic CaR transcript(s) was also detected by RT-PCR (Fig. 1*B*), performed using total RNA from LnCaP and PC-3 cells followed by sequence analysis of the PCR products.

These two prostate cancer cell lines also express CaR protein as assessed by immunocytochemistry (Fig. 2) and Western blot analysis (Fig. 3) performed using an affinity-purified, anti-CaR antiserum (4637). As assessed by Western analysis, the levels of CaR protein expression in LnCaP and PC-3 cells were substantially lower than in the positive controls, HEKCaR cells and bovine parathyroid cells. They are not dissimilar, however, from those in several other types of cells in which we have shown that the CaR is expressed and modulates various biological responses, such as regulation of Ca^{2+}-activated K^+ channels (9).

$[Ca^{2+}]_o$ and the polycationic CaR agonists neomycin and spermine each stimulated PTHrP secretion from LnCaP and PC-3 cells in a dose-dependent manner (Fig. 3*A*), with maximal stimulation occurring at 7.5–10 mM $[Ca^{2+}]_o$. The levels of $[Ca^{2+}]_o$ in the vicinity of resorbing osteoclasts are thought to be many times higher than the level of systemic $[Ca^{2+}]_o$ (i.e., as high as 8–40 mM) (36). Therefore, in the bony microenvironment, metastatic prostate cancer cells will likely encounter levels of $[Ca^{2+}]_o$ at least as high as those used in the present studies. Our results are consistent with those in other cell types exhibiting high $[Ca^{2+}]_o$-evoked PTHrP secretion, including normal keratinocytes (22), normal cervical epithelial cells (28), oral squamous cancer cells (31), JEG-3 cells (21), and H-500 rat Leydig cells, a model of humoral hypercalcemia of malignancy (34). The molecular mechanism underlying $[Ca^{2+}]_o$-stimulated PTHrP secretion in these cell types, however, is not clear. Our data suggest that the CaR is the likely mediator of this effect in PC-3 cells, because the receptor is clearly expressed in this cell line and PTHrP secretion is stimulated not only by elevated levels of $[Ca^{2+}]_o$ but also by the polycationic CaR agonists neomycin and spermine. Furthermore, adenovirus-mediated infection of the PC-3 cells with a dominant negative CaR (R185Q) (2) attenuated and right-shifted high $[Ca^{2+}]_o$-stimulated PTHrP secretion, providing additional strong evidence for mediation of this action by the CaR. Others have successfully utilized transfection of CaR-expressing cells with a different dominant negative CaR construct (R795W) to document the CaR's involvement in other biological responses (30).

On the basis of the present study on PC-3 cells and in two breast cancer cell lines (35), our findings have clear implications for the existence of a feed-forward mechanism involving prostate cancer cells metastatic to bone. When prostate and breast, and possibly other, cancers metastasize to the skeleton and induce PTHrP-mediated osteolysis, this will lead to high local levels of $[Ca^{2+}]_o$ within the bony microenvironment owing to PTHrP-stimulated bone resorption with or without associated systemic hypercalcemia. These high levels of $[Ca^{2+}]_o$ will elicit further PTHrP secretion from the cancer cells, thereby exacerbating the osteolytic disease. Guise and Mundy (18) have provided strong evi-

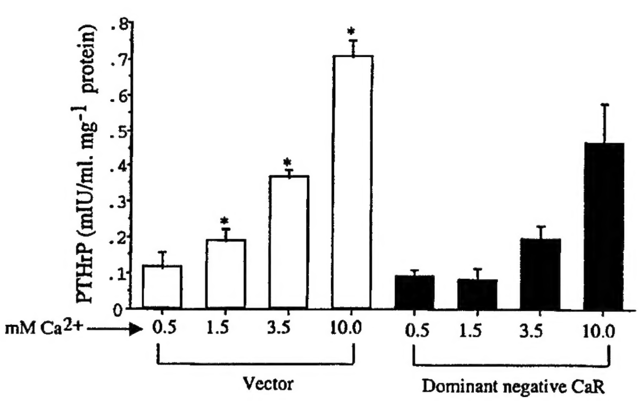

Fig. 5. Attenuation of high $[Ca^{2+}]_o$-stimulated PTHrP secretion from PC-3 cells infected with a dominant negative CaR. Open bars show PTHrP secretion in response to elevated levels of Ca^{2+} by PC-3 cells infected with the empty adenoviral vector; solid bars show the attenuation of high $[Ca^{2+}]_o$-stimulated PTHrP secretion in the cells infected with the dominant negative CaR. *Significant inhibition of PTHrP secretion from the dominant negative vs. vector-infected cells at the indicated level of $[Ca^{2+}]_o$. Similar results were observed in another experiment carried out using the identical experimental protocol.

dence for the existence of a similar feed-forward mechanism involving the action of TGF-β released from bone on PTHrP-secreting breast cancer cells. Indeed, we have shown that TGF-β_1 increases PTHrP secretion from PC-3 cells and have also demonstrated that TGF-β_1 produces at least an additive increase in the stimulation of PTHrP secretion by high $[Ca^{2+}]_o$. The mechanism for this effect is not clear but might involve TGF-β_1-induced upregulation of the expression of the CaR or its signaling pathways and/or of the level of expression of the PTHrP gene, thereby increasing the amount of PTHrP available for secretion in response to an elevated level of $[Ca^{2+}]_o$. Because $[Ca^{2+}]_o$ and TGF-β are both released from the bone matrix during bone resorption induced by PTHrP, they are both available to elicit further PTHrP secretion. In effect, both could cooperate to generate a vicious cycle of tumor-induced bone resorption begetting further bone resorption in the setting of skeletal metastases of prostate (or breast) cancers. The beneficial actions of bisphosphonates on the skeletal complications of metastatic breast cancer and on the incidence of new metastases (17, 32, 41) could result, at least in part, from reductions in the local concentrations of both $[Ca^{2+}]_o$ and TGF-β as a result of decreased bone resorption.

In addition to its potential role in stimulating PTHrP secretion from prostate cancer cells metastatic to bone, the CaR could also impact on tumor progression, osteolysis, and, in some cases, hypercalcemia by modulating the proliferation and/or apoptosis of tumor cells. Recent studies have shown that CaR activation stimulates proliferation in several cell types, including rat-1 fibroblasts (30). In PTHrP-producing tumors, the CaR could potentially increase proliferation directly and/or indirectly by enhancing PTHrP secretion. Indeed, PTHrP has been shown to stimulate the proliferation of H-500 rat Leydig cells in vitro and to increase the rate of tumor growth in vivo when H-500 cells are implanted subcutaneously in rats (33). The CaR also protects some cells against apoptosis, as we have shown recently for AT-3 rat prostate cancer cells and CaR-transfected, but not nontransfected, HEK-293 cells (29). Therefore, high $[Ca^{2+}]_o$-evoked, CaR-mediated stimulation of proliferation and/or inhibition of apoptosis of prostate cancer cells metastatic to bone could clearly contribute to the progression of tumor growth and potentially render the tumor cells resistant to therapy.

In summary, high $[Ca^{2+}]_o$-evoked, CaR-mediated PTHrP secretion could clearly contribute to the excessive bone resorption recently recognized to be an important complication of prostate cancer metastatic to bone. If, as in PC-3 cells, the CaR modulates PTHrP secretion in other prostate cancer cells, then the use of CaR antagonists (15) with some degree of specificity for prostate and other types of cancer cells that metastasize to bone and produce PTHrP and, therefore, osteolysis could potentially offer substantial therapeutic benefits in this setting.

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